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Complete development and multiplication of *Cryptosporidium hominis* in cell-free culture.

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Abstract

The present study reports for the first time the completion of the life cycle of *C. hominis* in cell-free culture and multiplication of the parasite via qPCR. Individual life cycle stages were characterised using *Cryptosporidium*-specific antibody staining (Sporo-Glo™) and fluorescent *in situ* hybridisation (FISH) staining on cultures inoculated with excysted oocysts and purified sporozoites. In both cultures, *C. hominis* successfully proliferated and completed its life cycle, however development in cultures inoculated with purified sporozoites lagged behind cultures inoculated with excysted oocysts. Some novel findings of the study include the visualisation of pairing and multiple associations between various developmental stages in a process similar to syzygy and the formation of *Cryptosporidium* stages (trophozoites and meronts) inside the oocysts without excystation. qPCR analysis revealed a 5-6-fold amplification of parasite DNA. Future studies are required to improve the amplification of the parasite. The present study confirms the suitability of this culturing model to support the growth and proliferation of *C. hominis* (which unlike *C. parvum,* cannot be readily cultured in small animal models) and will greatly assist in our understanding of the developmental biology of *Cryptosporidium*, its position within the Apicomplexa and its relationship to gregarine protozoa.

Keywords: *Cryptosporidium hominis,* Cell free culture, Excysted oocysts, Purified sporozoites, Sporo-Glo™, FISH, qPCR.

1. Introduction

The protozoan parasite *Cryptosporidium* is a significant entero-pathogen of both immunocompetent and immunocompromised vertebrate hosts world-wide (Xiao, 2009). There are currently 21 valid species of *Cryptosporidium* and numerous genotypes, many of which probably represent individual species (Xiao, 2009). The majority of cryptosporidial infections in humans are caused by *C. parvum* and *C. hominis*, with *C. meleagridis* emerging as the third most common species to cause infection (Cama et al., 2003; Xiao and Feng, 2008). *Cryptosporidium* species infect the intestinal or respiratory epithelial cells resulting in self-limiting diarrhea or pulmonary cryptosporidiosis in immunocompetent persons. However, immunocompromised individuals can experience severe chronic diarrhea and respiratory cryptosporidiosis that frequently leads to wasting and sometimes death (Xiao and Feng, 2008). Currently, nitazoxanide (NTZ) is approved for treatment of cryptosporidiosis in children and immunocompetent adults in the U.S.A., however NTZ is not effective without an appropriate immune response and is therefore ineffective for the treatment of immunocompromised individuals (Gargala, 2008).

Upon ingestion by a suitable host, the sporulated oocysts, which contain 4 sporozoites, excyst and adhere directly to the intestinal epithelial cells of the host. Cell invasion by the sporozoite is followed by intracellular development to a trophozoite. The trophozoites aggregate and undergo two merogony stages. Asexual replication occurs by re-infection of merozoites produced from meront I. Sexual development is initiated from merozoites from meront II. Type II merozoites are released and re-infect neighbouring cells were they develop into microgametes and matures into a zygote, which undergoes further development into an oocyst. Two types of oocysts are released: thick-walled

oocysts, which are excreted in the faeces or thin-walled oocysts for endogenous re-infection (auto-infection) (Barta and Thompson, 2006; Hijjawi, 2009).

Recent developments in the *in vitro* cultivation have revealed that *C. parvum* can complete its life cycle in media devoid of host cells (Hijjawi et al., 2004), which highlights the paucity of knowledge about the developmental biology of this parasite. This finding supports other biological and phylogenetic analysis (Bull et al., 1998; Carreno et al., 1999; Hijjawi et al., 2002; Leander et al., 2003; Rosales et al., 2005) suggesting that *Cryptosporidium* has a closer affinity with gregarines than with the coccidia. A recent study has reported the failure of *C. parvum* to propagate in host cell-free culture suggesting that this method may have to be modified before easy reproducibility can be obtained (Girouard et al., 2006). However, more recently, multiplication *C. parvum* DNA from cell cultures in the absence of host cells has been reported (Zhang et al., 2009).

Life-cycle stages of the parasite in cell-free culture are difficult to visualise as they are small and are dispersed throughout the medium and this has undoubtedly contributed to the problems in identifying life-cycle stages experienced by researchers who have attempted to use this method. A recent study compared three specific and three non-specific techniques for visualising *C. parvum* life-cycle stages in cell-free culture and reported that a combination of Sporo-Glo™ and Crypto Cel staining produced the best results and should allow easier and more reliable identification of life-cycle stages in cell-free culture in the future (Boxell et al., 2008).

In the present study, we report for the first time the characterisation of the life cycle stages of *C. hominis* in cell-free culture using a variety of stains and multiplication

of the parasite using qPCR. The stains used include the *Cryptosporidium* specific fluorescent antibody Sporo-Glo™ (Waterborne™ Inc., New Orleans, USA) and fluorescent *in situ* hybridisation (FISH) using a *Cryptosporidium* specific rRNA oligonucleotide probe (Vesey et al., 1998) and one non-specific dye; the protein dye Texas Red® (TR) (Invitrogen, USA), to provide more evidence for the development of the *Cryptosporidium* life cycle in cell-free culture.

2. Materials and methods:

2.1. Cryptosporidium genotyping and purification

The *C. hominis* isolate used in the present study was obtained from a local diagnostic laboratory from an infected human patient from Western Australia and genotyped by sequence analysis of the 18S rDNA as previously described (Ryan et al., 2003). The sample was purified using ether extraction and a Ficoll® density gradient as previously described (Meloni and Thompson, 1996). Purified oocysts were stored in phosphate buffered saline (PBS) and antibiotics (100 IU/ mL penicillin G, 0.1 µg/ mL streptomycin and 2.5 µg/ mL amphotericin B) at 4°C for a maximum of 2 weeks before use.

2.2. Bleaching and excystation of oocysts

Purified *C. hominis* oocysts were bleached prior to use by placing them into sterile water containing 0.25% sodium hypochlorite for 30 minutes at room temperature. The suspension was subsequently centrifuged at 2,000 x g for 8 minutes at room temperature. Oocysts were resuspended in acidic water (pH 2.5-3) containing 0.5% trypsin/EDTA to stimulate excystation and incubated at 37°C for 30 minutes with mixing every 5 min. Thereafter, the excystation suspension was centrifuged at 2,000 x g for 8 minutes and resuspended in sterile water. As a control, intact but heat inactivated oocysts (incubated at 90°C for 60 min.) were also processed for cell-free cultivation and were stained using the stains described below.

2.3. Filtration of excysted oocysts to purify sporozoites.

Following excystation of oocysts, the suspension was re-eluted in 5 mL of maintenance media and incubated at 37°C in a water bath for at least two hours to allow the full release of sporozoites from the oocysts. This suspension was then filtered through a syringe filter unit (Pall Scientific, New York, U.S.A.) containing a 2 µm Isopore™ membrane filter (Millipore, Massachusetts, U.S.A), to remove all oocyst shells and un-excysted oocysts, leaving a pure suspension of sporozoites. Following filtration, samples were checked by microscopy to ensure that they were oocyst-free and sporozoites were enumerated using a haemocytometer. As a control, intact but heat inactivated purified sporozoites (incubated at 70°C for 30 min.) were also cultivated and stained as described below.

2.4. Cell free cultivation.

Each well on a 48 well culture plate (Greiner Bio-One, CELLSTAR), contained 1.5 mL of cell free maintenance media (RPMI-1640) containing 0.03 g l-glutamine, 0.3 g sodium bicarbonate, 0.02 g bovine bile, 0.l g glucose, 25 µg folic acid, 100 µg 4-aminobenzoic acid, 50 µg calcium pantothenate, 875 µg ascorbic acid, 1% fetal calf serum (Invitrogen), 0.36 g HEPES buffer, 10,000 U penicillin G and 0.0l g streptomycin per 100ml, adjusted to pH 7.4 as previously described by Hijjawi et al., (2004). Wells were inoculated with a 10 µl suspension of excysted *C. hominis* oocysts or purified sporozoites (~1 x 10^4/excysted oocysts or sporozoites/well) and were mixed thoroughly to ensure each 10 µl aliquot contained approximately the same number of oocysts or sporozoites. The layer of coagulated newborn calf serum under the culture medium, which was included in the

initial publication by Hijjawi et al., (2004) was not included in the present study, as it did not increase parasite DNA by qPCR. Cultures were incubated at 37°C, 5% CO_2 for 9 days and were inspected daily under an inverted light microscope (Olympus IMT-2) at magnifications ranging from 100X – 400X to check for the presence of life cycle stages.

2.5. Preparation of parasites for staining and qPCR

Samples were removed after 24 hrs, 48 hours, 3 days, 5 days, 7 days and 9 days post inoculation for staining and qPCR analysis. For all staining methods and for qPCR, each 48 well culture plate was trypsinised for 20 minutes to lift any parasites that were stuck to the plates and then the contents of 4 wells were removed, pooled and centrifuged at 2,000 x g for 8 minutes to concentrate parasites. The supernatant was removed down to approximately 200 µl and the pellet resuspended and placed into a 1.5 ml centrifuge tube.

2.6. Sporo-Glo™ staining

A working stock solution of 1x Sporo-Glo™ (Waterborne, New Orleans, USA) antibody reagent was prepared following manufacturer's instructions and 50 µl of this preparation was added to the *C. hominis* sample followed by 45 minute incubation at 37 °C. Following incubation, PBS was added to make the volume up to 1.5 ml, the tubes were centrifuged at 2,000 x g for 8 minutes and the supernatant removed. Samples were washed again in 1 x PBS and the supernatant was removed and the pellet resuspended in 20µl PBS for examination by microscopy.

161 *2.7. Crypto Cel staining.*

162 Crypto Cel (Cellabs, Sydney, Australia) antibody reagent (25µl) was added to a
163 tube containing *C. hominis* parasites (see section 2.5), mixed and incubated at 37°C for
164 30 min. The volume was then made up to 1.5 ml using 1 x PBS. Tubes were centrifuged
165 at 2,000 x g for 8 min. and the supernatant removed. The pellet was washed again in 1 x
166 PBS and re-suspended in 50 µl 1 x PBS for examination by microscopy.
167
168 *2.8. Fluorescent in situ hybridisation (FISH)*
169 A previously published *Cryptosporidium*-specific oligonucleotide probe directed
170 against 18S rRNA and fluorescently labeled with Cy3 was utilised in this study; Cry1 (5'-
171 CGG TTA TCC ATG TAA GTA AAG-3') (Vesey et al., 1998). A second probe,
172 conserved for all bacteria EUB338 (Amann et al., 1990) was used as a negative control.
173 Hybridisation was conducted as previously described and the hybridisation buffer was
174 prepared in DEPC treated water to ensure the absence of residual RNAses (Vesey et al.,
175 1998). Probe concentrations were 100 pmoles/µl.
176
177 *2.9. Texas Red®-X succinimidyl ester (TR) staining*
178 A 10 mM stock solution of TR (base dye) (Invitrogen, USA) was prepared in
179 0.5% dimethylsulfoxide (DMSO) and then further diluted 1:500 in deionised water to
180 make a 20 µM working stock. An equal volume of 20 µM TR was added to the *C.*
181 *hominis* life cycle stage suspension. Samples were incubated at 37°C for 30 minutes, and
182 washed twice in 1 x PBS as described above.
183

184 *2.10. Microscopy and Imaging*

185 Cultures were examined daily using an inverted light microscope (Olympus IMT-
186 2) at magnifications ranging from 100X – 400X to check for the presence of parasites and
187 the life cycle stages represented and also to check for evidence of contamination. Wet
188 mounts of samples were stained as described above and then analysed using an Olympus
189 BX51 microscope and appropriate fluorescence filters and DIC optics. Images were
190 captured using an Olympus DP70 digital camera and associated imaging software.

191

192 *2.11. DNA extraction and qPCR analysis*

193 DNA was extracted from all samples using a MoBio PowerSoil™ DNA isolation kit (MO
194 BIO, Carlsbad, California, USA). This was performed in triplicate (i.e. 3 x 4 pooled
195 wells). qPCR was performed in triplicate using a *Cryptosporidium* diagnostic locus
196 unique to *Cryptosporidium* (Cgd3_440 which spans positions 52052 - 53389 of contig
197 AAEE01000004 which maps to chromosome 3) as previously described (Yang et al.,
198 2009). Heat-inactivated controls were analysed in the same manner. A standard curve
199 was constructed using 6 triplicates of genomic DNA extracted from known numbers of
200 oocysts and serially diluted at a 1:9 ratio, calibrated to correspond to 1-10,000 oocysts.
201 Statistical analysis was performed using SPSS 11.0 (Statistical Package for the Social
202 Sciences) for Macintosh OS X (SPSS inc. Chicago, USA) to determine if there was a
203 statistically significant increase in parasite DNA compared to heat-treated controls.

204

205 **3. Results**

206 In order to confirm the presence of various developmental stages of *C. hominis* in
207 cell-free culture, images for individual stages that developed from initial inoculations
208 with excysted oocysts and purified sporozoites were captured using the *Cryptosporidium*
209 specific polyclonal antibody Sporo-Glo™ and the Cry1 FISH probe. The negative control
210 bacterial FISH probe (EUB338) did not bind to any of the life cycle stages (data not
211 shown). Parasites in different life cycle stages for cultures inoculated with excysted
212 oocysts and purified sporozoites were compared using microscopy and qPCR. The purity
213 of the sporozoite preparation was assessed to be 100% (i.e. no oocysts) as determined by
214 microscopic examination and counting of sporozoites by haemocytometer.
215
216 *3.1. Life cycle stages of C. hominis in cultures inoculated with excysted oocysts*
217 The rate of excystation was variable as some oocysts excysted immediately
218 (Figure 1a) and some remained unexcysted for several days in cell-free culture.
219 Trophozoites (Figure 1b) appeared 24 hours post-inoculation of excysted oocysts into
220 cell-free cultures, which were consistent with previous studies (Hijjawi et al., 2002;
221 Hijjawi et al., 2004). Trophozoites were circular to oval, uni-nuclear stages, which
222 developed into meronts (Figure 1e). Trophozoites appeared to aggregate together in
223 groups of two or more (Figure 1b, 1c), and occasionally more than 20 trophozoites,
224 resulting in the development of the meront stage (Figure 1e). Aggregation of trophozoites
225 was also observed in HCT-8 cultures (Figure 1d) where multiple sporozoites penetrated
226 one cell and developed into trophozoites (Hijjawi et al., 2002, 2004). Trophozoites were
227 also observed pairing laterally together in a process similar to syzygy in gregarines,

228 which is defined as association of gregarine protozoa end-to-end or in lateral pairing
229 without sexual fusion (Figure 1b, 1f). Interestingly, the transformation of sporozoites to
230 trophozoites was also observed within unexcysted oocysts (Figure 1g), resulting in the
231 formation of meronts inside the oocysts without excystation (Figure 1h).

232 Meront stages were observed 48 to 72 hours post inoculation in cell-free culture
233 and consistent with a previous study (Hijjawi et al., 2004), two morphological types of
234 meronts were observed; meront I and meront II. Meront I were almost transparent grape-
235 like aggregates and were difficult to detect using Nomarski interference microscopy, but
236 were readily detected using FISH (Figure 1i) and Sporo-Glo™ (not shown). Meronts
237 varied in size according to the original number of trophozoites, which were originally
238 aggregated together. Meront II can be differentiated from meront I by the presence of
239 thick membrane around it which makes it impossible to identify its internal structure and
240 any processes which occurs inside it. Some of meront II stages formed a rosette-like
241 pattern (Figure 1j) and others were irregular in size and had different sizes and shapes
242 (not shown). Merozoites released from meront I and II varied in size and shape.
243 Merozoites released from meront I appeared to be actively motile and mostly circular,
244 however, merozoites released from type II meronts were bigger in size and sluggish,
245 either broadly spindle-shaped with pointed ends, or rounded to polymorphic and had a
246 thick membrane (Figure 1k).

247 Sexual stages (microgamonts and macrogamonts) were observed 5 days post-
248 inoculation in cell-free culture. As described in previous studies (Current and Reese,
249 1986; Hijjawi et al., 2004), microgamonts and macrogamonts appeared to form from type
250 II merozoites. Macrogamonts were similar in size to oocysts with a large single nucleus

(Figure 1l). Microgamonts, containing microgametes, occupied most of the cell around the residuum (not shown). Stages resembling zygotes/ unsporulated oocysts were also observed with a large undifferentiated nucleus (Figure 1m). After day 5 a mixture of all *C. hominis* developmental stages including trophozoites, meront I and II, gametocytes and oocysts were observed since the development of this parasite *in vitro* is not synchronized. Only oocysts were observed in the heat-treated controls. No progression to other life cycle stages was detected.

3.2. Life cycle stages of C. hominis in cultures inoculated with purified sporozoites

The life-cycle and all the described developmental stages described earlier in section 3.1 for *C. hominis* cultures inoculated with excysted oocysts was similar for those cultures inoculated with purified sporozoites (Figure 2), with the exception that development of life-cycle stages appeared delayed, with the later stages appearing much later than for cultures inoculated with excysted oocysts. Trophozoites appeared 24 hours post-inoculation of purified sporozoites into cell-free cultures and appeared to from aggregates of two or more (Figure 2b). As with cultures from excysted oocysts, aggregates up to 20 or more trophozoites were observed (Figure 2c), which resulted in the development of the meront I stages, which were not visualised until day 4 (Figure 2d) and meront II on day 5 (Figure 2e). On day 7, sporulated oocysts were observed (Figure 2f-g). Oocysts were also observed on day 8 and 9 (Figure 2h). No life-cycle stages or structures resembling life-cycle stages were detected in heat-treated purified sporozoite controls.

3.3. qPCR analysis of cultures inoculated with excysted oocysts, purified sporozoites and heat-inactivated controls.

qPCR analysis indicated an approximately 6.3-fold amplification of the parasite DNA for cultures inoculated with excysted oocysts over a 9 day period and a 5.9-fold amplification for cultures inoculated with purified sporozoites over the same period (Figure 3). A t-test revealed a significant ($p < 0.01$) increase in parasite DNA between days 5-9 compared to heat-treated controls. It was also evident that, similar to the delay observed in microscopic analysis of cultures inoculated with purified sporozoites, qPCR amplification of parasite DNA also appeared delayed, with no increase in DNA until day three (Figure 3). No amplification was seen in heat-inactivated controls (Figure 3).

4. Discussion

The present study reports for the first time the completion of the life cycle of *C. hominis* in cell-free culture and demonstration of multiplication of parasites using qPCR. In many previous studies, the observation of unsporulated /mature oocysts in any artificial culture system for *Cryptosporidium* provides definite proof for the completion of the life cycle of this parasite *in vitro* (Current and Reese, 1986; Hijjawi et al., 2003). The completion of *Cryptosporidium* life cycle by observing the production of oocysts was reported in cell-free culture (Hijjawi et al., 2004) as well in cell culture models using different cell lines such as Caco-2 cells, MDBK and HCT-8 cell lines (Buraud et al., 1991; Villacorta et al., 1996; Hijjawi et al., 2001; 2002). During the present study, the presence of *C. hominis* oocysts were confirmed in cell-free cultures inoculated with purified sporozoites by the demonstration of oocyst wall staining using Crypo Cel (a

297 monoclonal antibody preparation directed against the oocyst wall) (Figure 2g).

298 *Cryptosporidium hominis* is an important species, which is restricted to humans
299 and in many countries is more prevalent than *C. parvum* (Xiao, 2009). Being able to
300 culture *C. hominis* in cell-free culture is of great significance since this species cannot be
301 readily cultured in small animal models, unlike *C. parvum* which can be passaged
302 through mice (Meloni and Thompson 1996). A gnotobiotic pig model has been
303 established for *C. hominis* (Pereira et al., 2002, Widmer et al., 2000) and *C. hominis* is
304 also reportedly capable of infecting and proliferating in immunosuppressed gerbils
305 (Baishanbo et al., 2005); however, this is beyond the facilities of many laboratories. Thus
306 an *in vitro* cell-free culture model will open new insights into our understanding of this
307 human specific parasite and will facilitate many studies regarding its developmental
308 biology, pathogenesis and drug susceptibility.

309 The identification of all *C. hominis* life-cycle stages using both antibody (Sporo-
310 Glo^{TM}) and RNA (FISH) staining methods that are specific for *Cryptosporidium* provides
311 further proof of the ability of *Cryptosporidium* to amplify and propagate in a culture
312 system devoid of host cells. In the present study, all life cycle stages of *C. hominis* with
313 the exception of the oocyst stage, which is not recognised by Sporo-Glo^{TM}, reacted
314 strongly with Sporo-Glo^{TM} and it was easy to visualise each individual stage. Sporo-
315 Glo^{TM} was particularly useful for detecting and identifying meront I stages as they are
316 almost transparent using Nomarski interference microscopy (Figure 1i and 2d) and
317 therefore very difficult to detect in cell-free culture. TR staining although non-specific
318 was also included as it produced bright and clear details of the morphology of life cycle
319 stages and particularly internal structures.

qPCR analysis revealed a 6.3-fold increase in DNA over a nine-day period for cultures inoculated with excysted oocysts and a 5.9-fold increase in DNA for cultures inoculated with excysted sporozoites. This is similar to a recent study, which reported an estimated 5.6-fold increase in *C. parvum* DNA concentration over a 5-day culture period (Zhang et al., 2009). Both microscopy and qPCR analysis indicated that cultures inoculated with purified sporozoites lagged behind those inoculated with excysted oocysts. The reason for this is unclear. It may be due to the fact that sporozoites are much less robust than oocysts and the stress of filtration followed by washing and centrifugation, may affect their viability. When cell-free cultures are inoculated with excysted oocysts, the sporozoites can escape gradually from the oocysts shells and adapt quickly to the culture medium, therefore their chances for survival and initiation of their life cycle would be maximised. The observations in the present study are also consistent with a previous study in which it was reported that although both sodium hypochlorite-stimulated oocysts and purified sporozoites were able to initiate infection in BS-C-1 cell lines, cultures inoculated with purified sporozoites were less efficient (Deng and Cliver, 1998).

In the present study, several unusual developmental pathways, some of which have been previously observed in *C. parvum* cell-free culture (Hijjawi et al., 2004; Hijjawi, unpublished) were visualised for *C. hominis*. These include multiple associations and pairing between various developmental stages, the demonstration of two morphologically distinct meronts (I and II) and the formation *Cryptosporidium* stages inside the oocysts without excystation. Lateral pairing of two stages (intermediates between trophozoites and sporozoites) similar to syzygy (Figure 1b and 1f) similar to the

process which occurs in gregarines was observed for *C. hominis*. Syzygy of various developmental stages early in development has previously been described for *C. parvum* and *C. andersoni* (Hijjawi et al., 2002; 2004 and unpublished). Soon after excysting from oocysts, some sporozoites approached each other in multiple numbers and aggregated together end to end, but the majority transformed into oval / circular trophozoites which then developed into meronts of different sizes (Figure 1e).

Another interesting finding in the present study was the visualisation of trophozoites and meronts developing inside the oocyst. A recent study has also reported that merozoites were released from oocysts directly during an incubation and excystation procedure without bleach treatment (Karanis et al., 2008). Why this occurs is not understood. It may be a protective process by which *Cryptosporidium* can increase its proliferative potential while it is still protected inside the oocyst. Further studies are required to better understand this phenomenon.

A recent study reported that Nomarski interference microscopy images of *C. parvum* stages cell-free culture in the publication by Hijjawi et al., (2004) were actually debris and contaminants (Woods and Upton, 2007). However, the use of the *Cryptosporidium* specific polyclonal antibody Sporo-Glo™, the Cry1 FISH probe which is specific for *Cryptosporidium* and the fact that the negative control bacterial FISH probe (EUB338) did not bind to any of the life cycle stages in the present study provides strong evidence that the stages observed are in fact *Cryptosporidium* life-cycle stages and not contaminants. In addition, amplification of DNA detected via a highly specific qPCR and the lack of DNA amplification or the presence of life cycle stages in heat-treated controls also confirms that the parasite is indeed undergoing development in cell-free culture.

366 The description of the complete development for two species of *Cryptosporidium*
367 (*C. parvum* and *C. hominis*) in cell free culture, highlights new information and
368 differences to earlier interpretations of the developmental biology and life cycle of this
369 parasite. The present results support phylogenetic studies suggesting that
370 *Cryptosporidium* spp. are more closely related to gregarines than to coccidia (Morrison
371 and Ellis, 1997; Carreno et al., 1999; Leander et al., 2003; Barta and Thompson, 2006).
372 Only a 5 to 6-fold amplification of parasite DNA was attained, indicating that parasite
373 multiplication was far from optimal and future studies need to concentrate on improving
374 the level of parasite multiplication.
375 Further research using cell free culture may unveil the capability of this intriguing
376 parasite to amplify under different conditions and provide more information on its
377 developmental biology. By combining current and future developmental and
378 phylogenetic studies of this parasite, a clearer understanding of its position within the
379 Apicomplexa and its closeness to the gregarines species can be achieved.
380

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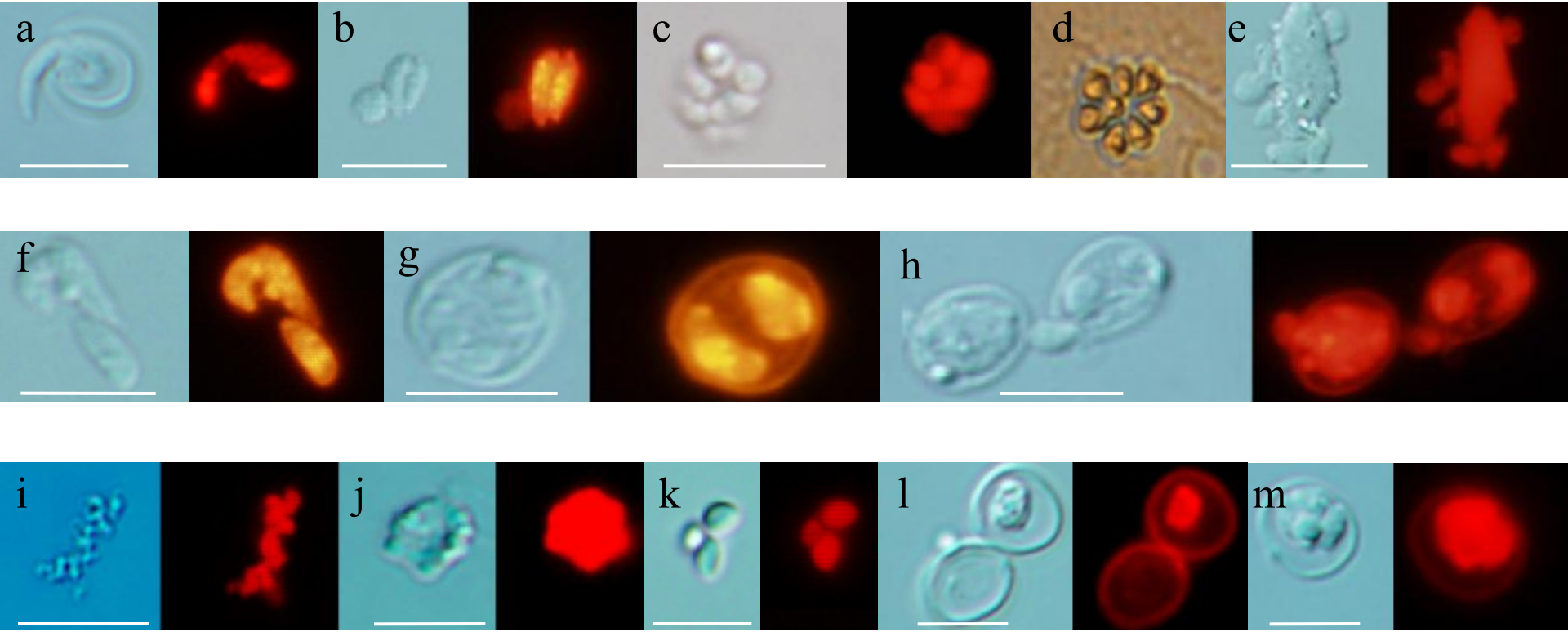
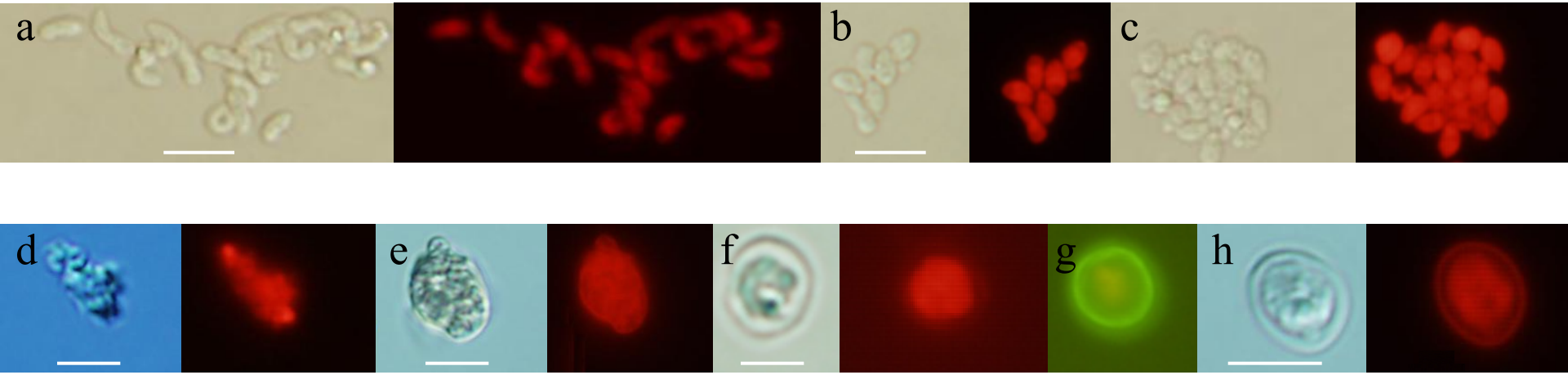
Figure 1. *Cryptosporidium hominis* life-cycle stages from cell-free cultures inoculated with excysted oocysts. a= excystation of sporozoites, Nomarski DIC (left) and stained with Sporo-Glo™ (right); b = two *C. hominis* trophozoites (intermediate form between trophozoite and sporozoite pairing together in a process similar to syzygy and a third circular one is also attached to the two, Nomarski DIC (left) and TR staining (right); c = pairing of multiple *C. hominis* trophozoites and (right) stained with Sporo-Glo™; d = multiple aggregation of trophozoites in HCT-8 cells similar to those observed in cell-free culture; e = development of a meront from trophozoites, Nomarski DIC (left) and Sporo-Glo™ staining (right); f = trophozoites pairing end to end in a process similar to syzygy, Nomarski DIC (left) and TR staining (right); g = trophozoites forming inside an oocyst without excystation, Nomarski DIC (left) and TR staining (right); h = aggregation of trophozoites and formation of meronts inside the oocyst stage, Nomarski DIC (left) and TR staining (right); i = meront I stage, Nomarski (left), FISH staining (right); j = meront II forming a rosette-like pattern 96 hrs post inoculation of *C. hominis* oocysts in cell-free culture, Nomarski DIC (left) and FISH (right), k = merozoites released from meront II which are characterized by being spindle in shape with pointed ends, Nomarski (left) and Sporoglo (right), l = macrogamont characterised by having a thick cell wall, similar in size to oocysts with one large nucleus, Nomarski DIC (left) and FISH (right), m = zygote and the start of sporogony, Nomarski DIC (left) and Sporo-Glo™ (right). Scale bar = 5 µm.

Figure 2. *Cryptosporidium hominis* life-cycle stages from cell-free cultures inoculated with purified sporozoites; a = purified sporozoites, b = pairing of *C. hominis* trophozoites, Nomarski DIC (left) and (right) stained with Sporo-Glo™; c = aggregation of trophozoites, in the process of developing into meronts, Nomarski DIC (left) and FISH staining (right); d = meront I stage, Nomarski (left), FISH staining (right); e = meront II stage, Nomarski (left), Sporo-Glo™ staining (right); f = oocyst day 7, Nomarski (left), Sporo-Glo™ staining (right); g = same oocyst stained with CryptoCel™; h = oocyst day 9, Nomarski (left), Sporo-Glo™ staining (right). Scale bar = 5 µm.

Figure 3. qPCR analysis of cultures inoculated with excysted oocysts, purified sporozoites and heat-treated controls over a nine day period.

Figure

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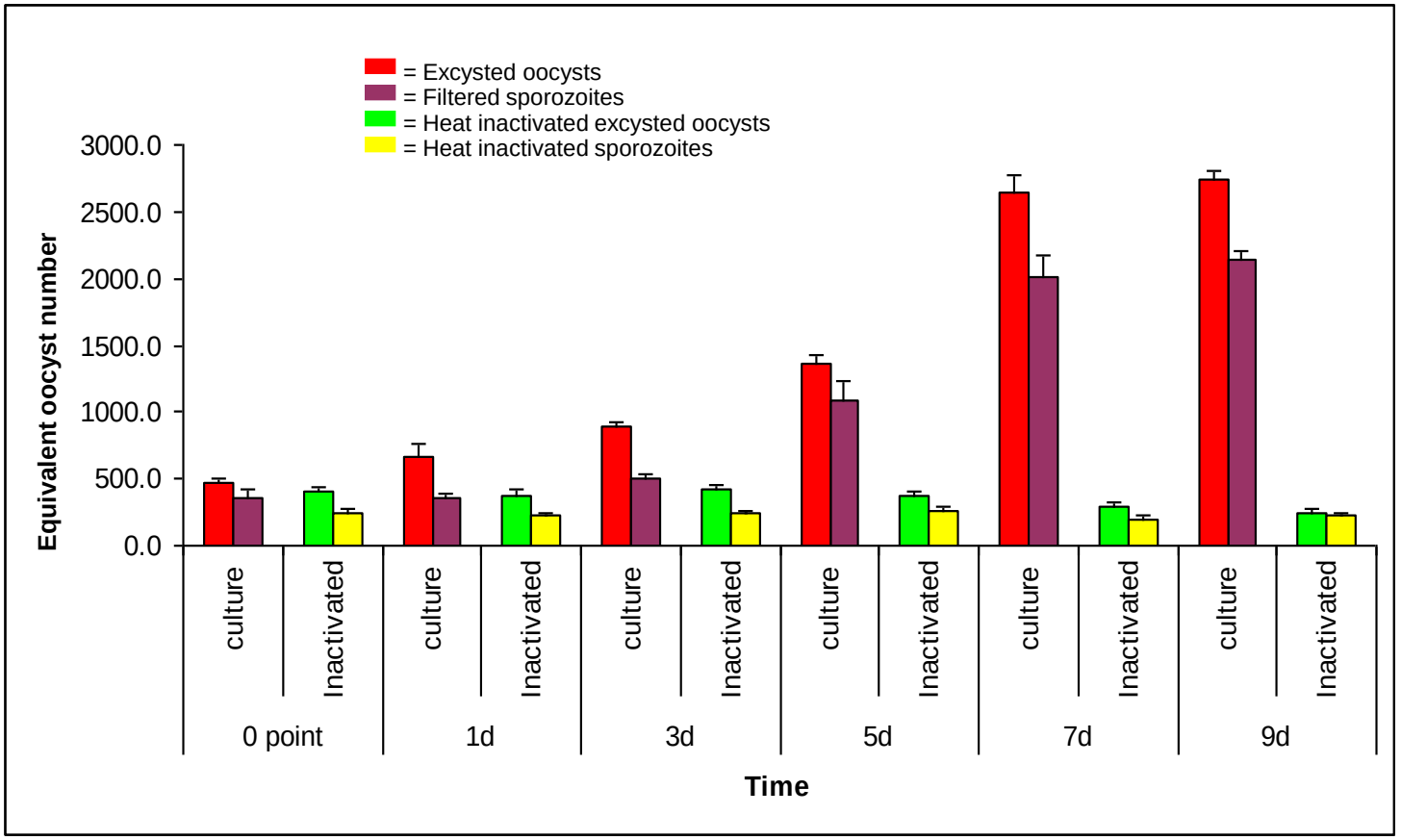
= Excysted oocysts
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= Heat inactivated sporozoites
Equivalent oocyst number
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
culture
Inactivated
0 point
1d
3d
5d
7d
9d
Time